ZOOLOGICAL SCIENCE 10: 763–772 (1993)

Endothelin-1 Causes Aggregation of Pigment in Teleostean Melanophores

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ABSTRACT—In many species of teleosts, and in zebrafish (*Brachydanio rerio*) in particular, endothelin-1 (ET-1) caused aggregation of pigment within melanophores in a dose-dependent manner. ET-1 appeared to act directly on the melanophores since denervated melanophores responded to the peptide quite similarly to the normally innervated cells. Alpha-adrenergic blockers, namely, phentolamine and tolazoline, and a muscarinic cholinergic blocker, scopolamine, did not interfere with the action of ET-1 on melanophores. Contrary to our expectations, BQ-123, an inhibitor of mammalian ET-1 receptors (ET_A receptors), did not block the action of ET-1 on melanophores. Presumably, teleostean ET receptors have very different pharmacological characteristics from those of mammalian species. In many fishes, ET may be involved in the subtle and delicate modification of hues and patterns of the integument that are associated with their elaborate and effective chromatic strategies for survival.

INTRODUCTION

Endothelin (ET) is a vasoconstrictive peptide that was first isolated from medium in which porcine vascular endothelial cells had been cultured [26]. ET very effectively induces the constriction of almost all vessels, both arterial and venous, of a number of mammalian species. There are, in fact, three isopeptides of ET, namely, ET-1, ET-2 and ET-3, and they have various biological effects on vasculature [13, 18].

To date, only few studies have been reported on the presence in and the effects of ETs on lower vertebrates. Uemura *et al.* [24] detected immunoreactive ET in the plasma of several lower vertebrates, including two teleost species. Kasuya *et al.* [16] described the presence of immunoreactive ET in the central nervous system of the medaka. Furthermore, it was recently shown that mammalian ET-1 causes contraction of the vasculature in the rainbow trout *Salmo gairdneri* (*Oncorhynchus mykiss*) [20]. Poder *et al.* [22] reported that ET-1 induced vasoconstriction of mesenteric arteries in a species of catfish *Ictalurus* (*Ameiurus*) *melas* and in other poikilothermic vertebrates. To date, there have been no well documented reports on the effects in fish of ETs on cells other than those of the vascular musculature. In the present study, therefore, we examined the effects of this peptide on the dermal melanophores of several species of fish. Since the chemical characterization of fish ETs has not been performed, we chose a readily available mammalian ET-1 as a representative ET.

Accepted June 14, 1993
Received May 27, 1993
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MATERIALS AND METHODS

Materials

Adult forms, without reference to sex, of the zebrafish (*Brachydanio rerio*), the rose bitterling (*Rhodeus ocellatus ocellatus*), the Nile tilapia (*Oreochromis niloticus*), the mailed catfish (*Corydoras paleatus*), the white cloud mountain minnow (*Tanichthys albonubes*), the common minnow (*Zacco platypus*), the black striped gudgeon (*Pungtungia herzi*), the medaka (wild type; *Oryzias latipes*) and the marble goby (*Oxyeleotris marmoratus*) were employed. These teleostean species were purchased from the local dealers in

Tokyo and in Chiba Prefecture, and they were reared in freshwater aquariums in our facilities for at least a week for acclimatization.

Scales with pieces of integumental tissue attached from all species named above were subjected to measurements of the responses of melanophores. Specimens were isolated from the dorso-lateral part of the trunk, except in the case of the mailed catfish *Corydoras,* in which the middle part of the mail, including the lateral line, was excised and carefully cleaned by discarding the debris of connective tissue components [14]. When we used Nile tilapias, split-fin preparations [1] were also employed. The specimens of skin were excised and prepared in a physiological saline solution for teleosts, which had the following composition (in mM): NaCl 125.3, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, (*R*)-(+)-glucose 5.6, Tris-HCl buffer 5.0 (pH 7.3).

Among the fish listed above, zebrafish were predominantly used for quantitative studies, because these fish were readily available, and reproducible results were consistently obtained if we focused on the melanophores around the center of a scale for measurements. In addition, since zebrafish can be conveniently employed for assessing the extent of water pollution, and since their eggs are frequently used for biotechnological studies which include efforts to obtain transgenic fish, we have begun to direct our attention to this species in particular. We expect that the outcome of our chromatic physiological studies of this species will provide useful information for future experiments with these fish.

The scales on the dorso-lateral surface of zebrafish were plucked out from the anterior part of the trunk for experiments. The melanophores are not located in the dermis between the epidermis and the bony scale in this fish, but are found in the thin dermal loose connective tissue below the scales. This situation resembles that in the dark chub, *Zacco temmincki* [10], and in some other cyprinids, for example, the topmouth gudgeon, *Pseudorasbora parva* (Fujii, unpublished observations). Without a covering of epidermis, which always forms a strong barrier to the diffusion of solutes, the melanophores are very accessible to stimulatory chemicals, and, indeed, they exhibit very high responsiveness to chemical stimulants.

Before experimentation, scales isolated from the medaka and the marble goby were stored in a refrigerator (4°C) for 30–60 minutes in a saline solution that lacked Ca^{2+} and Mg^{2+} ions. The recipe of the solution was as follows (in mM): NaCl 130.7, KCl 2.7, (*R*)-(+)-glucose 5.6, ethylenediamine tetraacetic acid (disodium salt; Dojindo Lab., Kumamoto, Japan) 1.0, Tris-HCl buffer 5.0 (pH 7.3). Then the epidermis was carefully removed with fine forceps.

In some experiments, responses of denervated melanophores were studied. Such melanophores were obtained by intraperitoneal injection of 6-hydroxydopamine (6-OHDA) [11]. Zebrafish, anesthetized by immersion in a 0.005% solution of MS-222, were injected with 6-OHDA (80 μg per gram body weight). The melanophores on a scale plucked from a fish that had been left overnight in an aquarium were tested to determine whether the cells had been denervated. When the melanophores were found to be refractory to an elevation in the level of K^+ ions in the medium, they were regarded as being denervated.

Recording of responses of melanophores

The physiological and pharmacological methods employed were basically the same as those described in previous reports [3, 21]. The photoelectric method for recording motile responses of melanophores was fundamentally identical to that described by Oshima and Fujii [21], but the electronic circuitry was somewhat modified by incorporation of integrated circuits of a new type, namely, CMOS chopper-stabilized operational amplifiers (MAX421; Maxim Integrated Products, Sunnyvale, CA). As a result, offset correction, which is a rather difficult procedure for novices, was not necessary for practical purposes (Fujii *et al.*, to be described elsewhere).

Light transmittance through a circular area of skin with a diameter of 150 μm was measured. In many cases, the area was sufficient to include the region occupied by only a single melanophore [21]. When smaller melanophores were densely distributed as in the scales of zebrafish, a few melanophores were included within each domain, and the net effects of their motile responses were

recorded. At the end of each series of measurements, a sufficiently strong solution of norepinephrine hydrochloride (NE; racemic modification; Sankyo, Tokyo) made up in the physiological saline was applied for a few minutes in order to bring about the full aggregation of pigment for reference. Usually a 50 μM solution of NE was strong enough for this purpose. The concentration of NE is expressed in terms of the sum of the concentrations of the active (*R*)-(−)- and inactive (*R*)-(+)-isomers. The full extent of the response to ET-1 was sometimes recorded before treatment of a specimen with norepinephrine, in particular when solutions of ET-1 at higher concentrations were employed. In all cases, the magnitude of the response was expressed as a percentage of the maximal response observed during the course of measurements, with the fully dispersed state taken as zero.

Drugs used

Chemical structures of teleostean ETs have not yet been determined. Therefore, a mammalian ET-1 (ET-1, human/porcine; Sigma Chemical, St. Louis, MO) was selected as a representative of the three known ET isopeptides because this peptide is known to produce definitive and pronounced contraction of vascular smooth muscles in many animal species, including some teleosts (cf. Introduction).

In addition to ET-1 and NE, the following drugs were employed: phentolamine mesylate (Ciba-Geigy, Basel), tolazoline hydrochloride (Sigma Chemical), acetylcholine chloride (Daiichi Seiyaku, Tokyo), scopolamine N-butylbromide (Yamanouchi Pharmaceutical, Tokyo), 3-aminobenzoic acid ethyl ester (methanesulfonate salt; MS-222; Nacalai Tesque, Kyoto) and 6-hydroxydopamine hydrobromide (Sigma Chemical). BQ-123-Na, an antagonist of ET receptors of the ET_A type, was a gift from Dr. M. Yano, Banyu Pharmaceutical Co, Tsukuba, Ibaraki. Its structure has been determined to be cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-) [12]. Stock solutions of these drugs were diluted with the physiological saline immediately before use.

Before we examined the effects of ET-1, K^+-rich saline was frequently applied to the preparations of skin to confirm that the melanophores exhibited normal responsiveness. We adopted stimulation by K^+ ions for this purpose since the induced aggregation of pigment was rapidly reversible, and the aftereffects of the stimulation were minimal. The responsiveness to K^+-rich medium also provided evidence that the cells were normally innervated, since the aggregation of pigment in response to K^+ ions is known to be induced via the release of neurotransmitter from sympathetic fibers [1]. In the present study, a saline solution containing 50 mM K^+ ions was employed. The concentration of Na^+ ions was appropriately decreased so that the final osmolarity was the same as that of the normal saline.

All physiological measurements were made at room temperature (20–25°C).

RESULTS

Effects of ET-1 on melanophores

Motile responses of melanophores to endothelin-1 (ET-1) were examined in the teleostean species. Most melanophores in preparations of skin from the fishes listed in Materials and Methods responded to ET-1 by the aggregation of their pigmented organelles, the melanosomes. The photomicrographs shown in Figure 1 demonstrate that ET-1 effectively induced the aggregation of melanosomes in the melanophores on the scales of the zebrafish. To confirm that the melanophores actually had normal motile activity, a K^+-rich saline solution was applied prior to the application of ET-1 (Fig. 1).

Among the fish studied, the Nile tilapia was an exception. The melanophores on the scales, as well as on the split-tail fin preparations, of this species were found to be refractory to ET-1. Furthermore, the melanophores on scales of the medaka and the marble goby were only found to be responsive to ET-1 after removal of the epidermis on the top of the loose connective tissue in which the melanophores were embedded.

Melanophores on scales of the zebrafish were employed for quantitative studies, as mentioned above. Typical photoelectric recordings of the responses to 50 mM K^+ ions and then to 10 nM

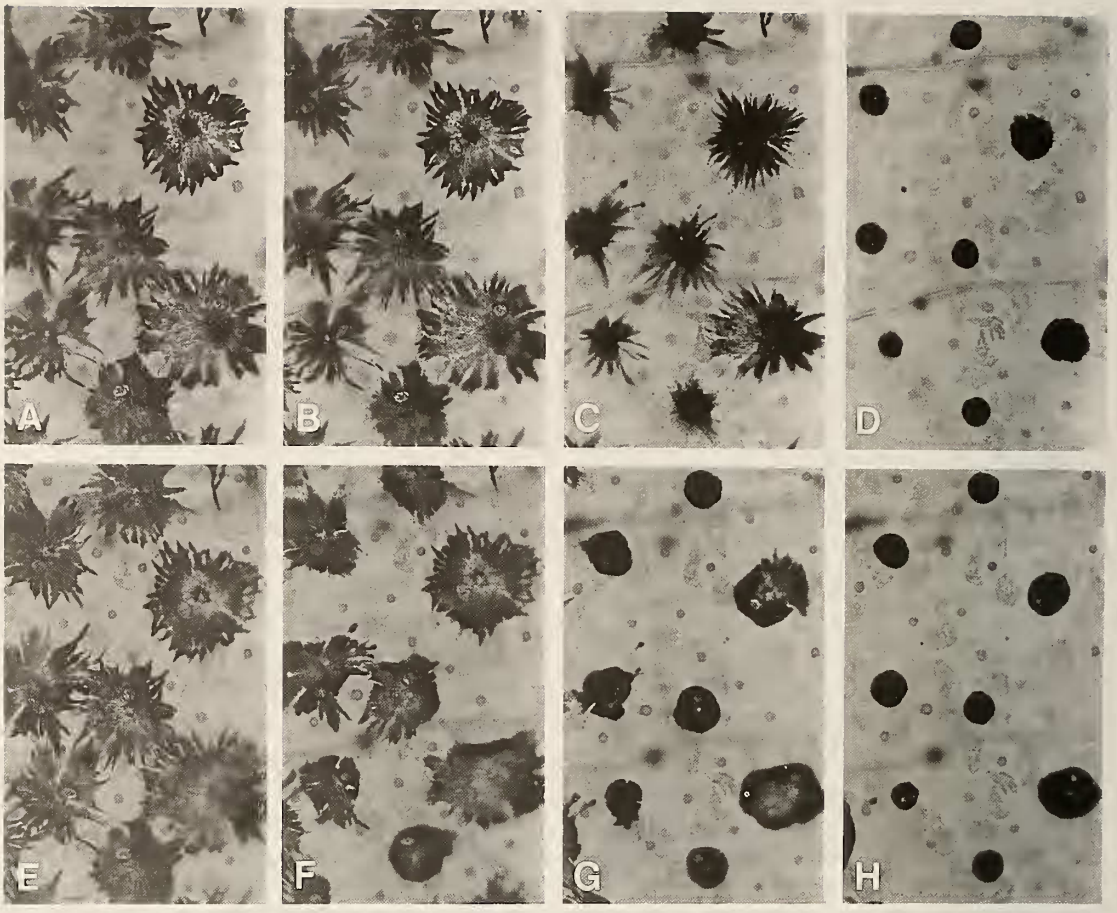

Fig. 1. Serial photomicrographs showing the effects of K^+-rich saline and mammalian endothelin-1 (ET-1) on the melanophores in a scale of the zebrafish (*Brachydanio rerio*). **A**: Equilibrated in physiological saline. Melanosomes are completely dispersed in the cells. **B, C** and **D**: 10, 20 and 90 sec, after the application of saline that contained 50 mM K^+ ions, respectively. Rapid aggregation of melanosomes is seen. **E**: Equilibrated again in physiological saline for dispersal of melanosomes. **F, G** and **H**: 60, 150 and 300 sec, after the application of 10 nM ET-1, respectively. Gradual aggregation of melanosomes into perikarya is clearly seen. ×200.

ET-1 of melanophores of a zebrafish are shown in Figure 2 (A and B). ET-1 induced the aggregation of melanosomes very effectively, although the rate of the response was rather low. We found that, in some melanophores, such as the ones for which responses are shown in panel A of Figure 2, the aggregation persisted for a long time. In other cases, by contrast, the aggregation was gradually reversed as shown in panel B of the same Figure. However, the maximal extent of the aggregation of melanosomes was very high when the concentration of ET-1 was higher than 10 nM.

The relationship between the concentration of ET-1 and the extent of the aggregation of melanosomes is shown in Figure 3. At first, we confirmed the responsiveness of melanophores by applying K^+-rich saline. After washing the scale with the primary saline, a solution containing ET-1 at one of various concentrations from 1 pM to 1 μM was applied. The sample was perfused with each solution for 10 min. The effect was found to be concentration-dependent, and the curve showing this relationship has a typical sigmoid shape. Apparent aggregation of pigment was detectable at the very low concentration of 100 pM, and the maximal level was attained at the concentration of

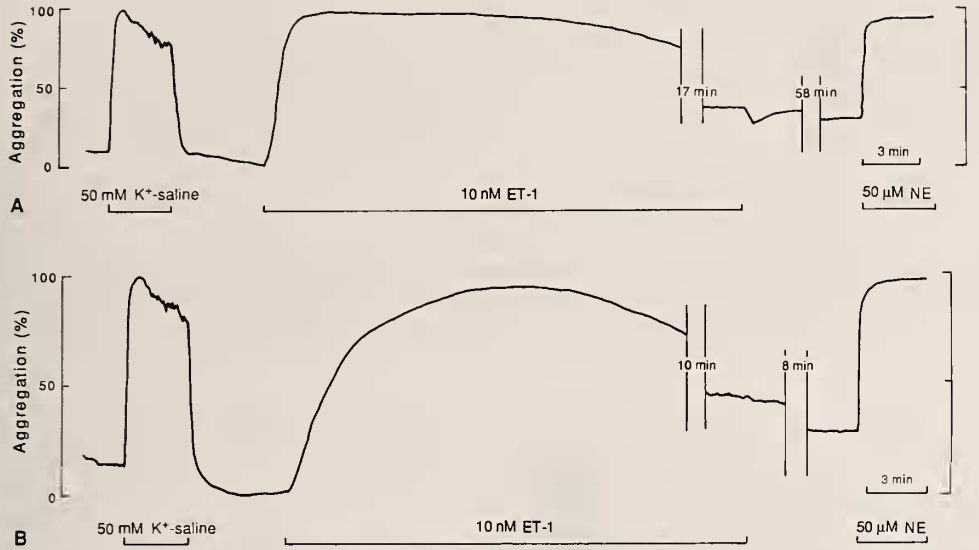

FIG. 2. Typical photoelectric recordings showing the responses of melanophores in a scale from a zebrafish to 50 mM K^+, 10 nM ET-1, and then to 50 μM norepinephrine (NE). **A**: Recording of a case in which the aggregation of pigment in response to ET-1 persisted for a long time. **B**: A case in which the aggregation was gradually reversed even in the presence of ET-1.

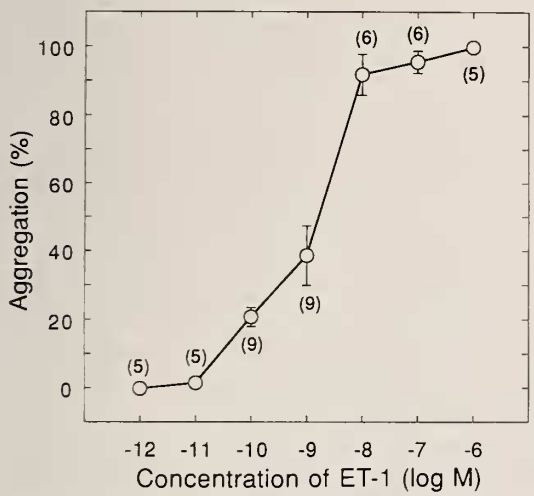

FIG. 3. Relationship between the concentration of ET-1 and the extent of the aggregation of melanosomes during 10-min application of the peptide to melanophores of the zebrafish. At the end of each series of measurements, a 50 μM solution of norepinephrine was applied for 3 min. Abscissa: concentration of ET-1 (logarithmic scale). Ordinate: magnitude of response as a percentage of the full aggregation of melanosomes attained during each series of measurements. Each point represents a mean value with SE (vertical bar). The numbers of measurements, on scales from different animals, are shown in parentheses.

1 μM.

It should be noted that, once the aggregation of pigment in melanophores had occurred in response to ET-1, the cells usually became refractory to the peptide (Fig. 4). However, even after exposure to ET-1, the melanophores were found to retain normal responsiveness to other chromatophore-regulating substances, including norepinephrine, that were commonly employed to induce the maximal aggregation of pigment at the end of each series of measurements.

Effects of ET-1 on denervated melanophores

The effects of ET-1 on denervated melanophores were examined. Results of a typical series of experiments to examine the consequences of denervation are shown in Figure 5. In the left part of the Figure, the absence of responsiveness of the melanophores to K^+ ions is shown. The refractoriness indicated that the melanophores were actually denervated. When 10 nM ET-1 was applied, marked aggregation of melanosomes took place.

Effects of several receptor antagonists

The effects of several drugs that are known to

Fig. 4. Typical recording of the responses of melanophores on a scale of a zebrafish, showing the melanosome-aggregating action of ET-1. Note that once the aggregation of pigment had been induced by the action of ET-1, the cells became almost completely refractory to the peptide even though their responsiveness to other pigment-motor substances, including norepinephrine (NE), was unchanged, as shown in the right part of the Figure.

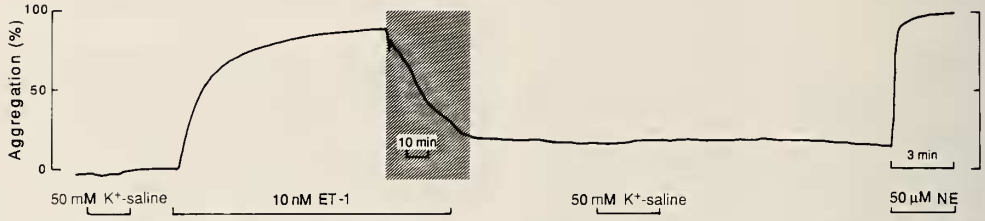

Fig. 5. Typical recording showing the effect of ET-1 on denervated melanophores of the zebrafish. The cells under study were confirmed as actually having been denervated by the absence of a response to an elevated concentration of K^+ ions (50 mM K^+). A solution of ET-1 was then applied, with resultant marked aggregation of melanosomes. The hatched part indicates the time during which the driving speed of the recording chart was reduced as indicated. NE: norepinephrine.

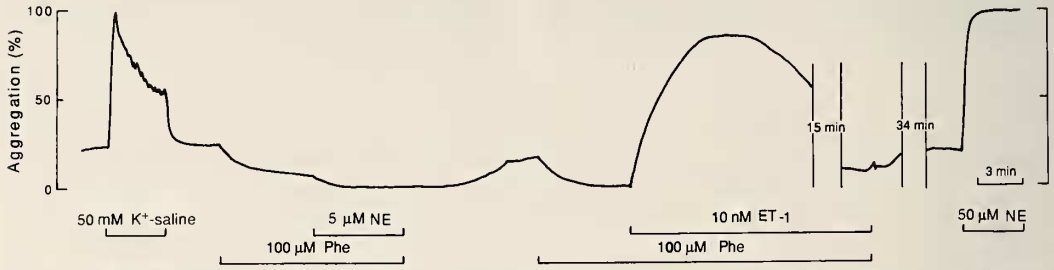

Fig. 6. Typical recording of the responses of melanophores of the zebrafish, showing the effect of an alpha-adrenolytic agent, phentolamine (Phe), on the melanosome-aggregating actions of norepinephrine and ET-1. While the action of norepinephrine (NE) was effectively antagonized, that of ET-1 was not affected by the blocker.

interfere with the receptors on chromatophores that mediate the aggregation of melanosomes were studied. As representative of alpha-adrenolytic agents, phentolamine and tolazoline were tested for their possible inhibitory effects on the melanosome-aggregating action of ET-1. As shown in Figure 6, phentolamine did not interfere with the action of ET-1. Tolazoline also lacked any inhibitory effects (data not shown).

The effect of a muscarinic cholinergic blocker, scopolamine, was then examined since melanophores of many siluriform catfish [15] and of a few cyprinids [9] are known to have cholinoceptors of the muscarinic type that mediate the aggrega-

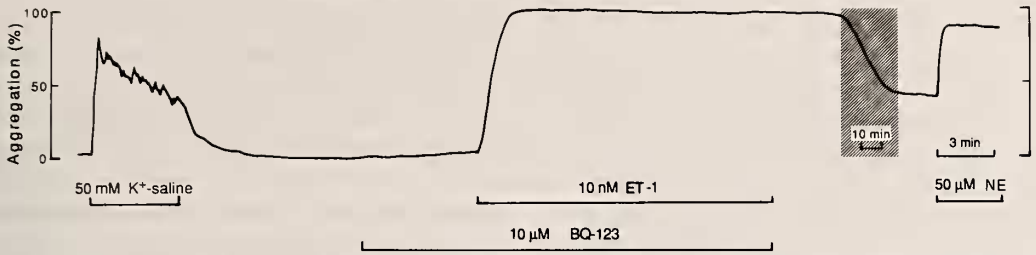

FIG. 7. Typical recording of the responses of melanophores, showing the absence of any effect of BQ-123, an inhibitor of ET_A receptors, on the melanosome-aggregating action of ET-1. Note the total ineffectiveness of the blocker. NE: norepinephrine. The hatched part indicates the time during which the driving speed of the recording chart was reduced as indicated.

tion of melanosomes. Scopolamine also had no effect on the action of ET-1 (data not shown).

Effects of an ET-receptor blocker

A recently developed blocker of mammalian ET_A receptors, BQ-123, was examined for its possible effects. Contrary to our expectations, the blocker did not have any inhibitory effect, even at very high concentrations (Fig. 7).

DISCUSSION

Yada *et al.* [25] reported that ETs promote the proliferation and melanization of human melanocytes. In the present report, we paid attention to the effects of this type of peptide on the motile activities of pigment cells and could find pigment-aggregating action of ET-1 for the first time. Namely, ET-1 at concentrations much lower than that we ever expected caused the aggregation of pigment in melanophores in preparations of skin excised from many of the teleost species examined. Since denervated melanophores responded in quite a similar manner to normally innervated cells, it seems clear that the peptide acted directly on the melanophores, not *via* an effect on the endocrine or nervous elements that can be found in the vicinity of the target cells.

In both innervated and denervated melanophores, ET-1 at concentrations as low as 100 pM induced the apparent aggregation of pigment, while the peptide at 10 nM normally induced a near maximal response. Thus, the action on melanophores seems not to be merely pharmacological, but to have physiological relevance. It may be safely proposed, therefore, that one or a few peptide species belonging to the ET family is involved in changes in integumental coloration *via* the control of chromatophores. Categorized as "paraneurons", vertebrate chromatophores, including melanophores, are of ectodermal origin. Thus, we have now demonstrated the effect of ET on cells of an ontogenetic origin other than the mesoderm, from which muscle cells originate exclusively.

Using a radioimmunoassay for ET-1, Uemura and her associates [24] detected an ET-like principle in the blood plasma of a number of vertebrates, including two species of teleosts, namely, the common Japanese conger *Conger myriaster* and the carp *Cyprinus carpio*. As a consequence, they suggested the general occurrence of peptides of this type throughout the vertebrates. The presence in the blood of the principles strongly suggests that they are the hormonal principles. Thus, it may rather safely be assumed that such peptides function in the endocrine regulation of teleostean chromatophores. We presume that they may be secreted from endothelial cells, as their original nomination signifies.

Using an immunocytochemical technique, Kasuya *et al.* [16] showed that such an ET-like substance was detectable in the hypothalamo-neurohypophyseal system and in the caudal neurosecretory system of the medaka *Oryzias latipes*. Thus, the neurohypophysis or urophysis might also be the endocrine gland involved in the synthesis of ET-like hormone(s). These various observations indicate that, in lower vertebrates, circulating ET may function as a hormone to

control certain target cells that naturally include vascular smooth muscle cells. Now, we can add the chromatophores to the list of possible target cells for ET-related compounds in fish. In addition to the neurohypophysis or the urophysis, we suggest that the chromaffin cells of adrenal tissue are another possible site in fish of secretion of ET since Sawamura *et al.* [23] recently indicated that, in mammals, ETs are also produced in chromaffin granules of adrenal chromaffin cells. In fish, incidentally, adrenal chromaffin cells that correspond to those of higher vertebrates are known to be present in the head kidney.

From the observations of the presence of an ET-like peptide in peripheral tissues of fish, Kasuya *et al.* [16] suggested that the peptide might function as a neurotransmitter or neuromodulator. Neurotransmitter substances applied extrinsically to a preparation of skin usually have an effect on chromatophores only at relatively high concentrations [3–5, 7, 14]. This observation may indicate that, for successful neurotransmission, rather high concentrations of the transmitter are needed at the surface of the postsynaptic membrane. It seems likely, therefore, that ET acts not as a neurotransmitter, but as a hormone or as a neuromodulator to bring about subtle adjustments of the motile activities of chromatophores that are under the coarse control of the principal pigment-motor cues [2, 6].

It is also possible that ET is secreted as a local hormone or an autacoid to affect localized chromatic changes, for example, changes in color patterns, by modulating the actions of known nervous and hormonal principles. In such a case, peripheral chromaffin cells scattered in the dermis might be candidates for the secretory cells in question [19]. Such a mode of endocrine action can be categorized as "paracrine" secretion.

To date, we still have no information about the molecular structures of ETs in fish. Given the much longer phylogenetic history of the class Osteichthyes, the diversity of their ETs should be even greater than that in mammals [13]. Thus, we can speculate that many teleostean ETs might have numbers and sequences of amino acids rather different from those of mammals because these two classes of vertebrates have evolved separately for more than 400 million years, even though they may initially have possessed common ancestral genes for ET. Nevertheless, as shown herein, human/porcine ET-1 acts very effectively on melanophores of many species of fish. This result indicates that ETs of fish may have molecular structures that are not so very different from those of mammals, notwithstanding the long history of divergence. In other words, ETs may be rather conservative peptides. Future attempts at cloning the genes for ETs from lower vertebrates should provide information about these issues.

In teleosts, sympathetic postganglionic innervation is primarily responsible for the aggregation of pigment in melanophores, and alpha-adrenoceptors are involved in this process [2, 3, 6]. In a few fish in the superorder Ostariophysi, to which zebrafish and some other species used in the present study belong, cholinoceptors of the muscarinic type are present that also mediate the aggregation of pigment [4, 7, 9, 15]. However, neither phentolamine, an alpha adrenolytic agent, nor scopolamine, a muscarinic cholinolytic agent, interfered with the action of ET-1. Therefore, it can be safely stated that the action of ET-1 is mediated neither by receptors in these categories, nor is it elicited by neurotransmitters liberated through the stimulation by ET-1 of the nervous elements that control melanophores.

The best-known hormonal agents that induce aggregation of pigment in melanophores are melanin-concentrating hormone (MCH) and melatonin [2, 6]. Effective specific antagonists for these principles are not yet been available. Thus, the possibility of the involvement of receptors for these hormones in the action of ET-1 cannot be ruled out completely. However, this possibility seems quite unlikely because the molecular structures of MCH and melatonin are quite different from those of ETs. Another possibility is that ET-1 acts to release such principles from their local storage sites, and that they, in turn, cause aggregation of melanosomes. Since such storage cells have not been shown to exist, the possibility can also be ruled out.

In the present study, BQ-123, a selective antagonist developed for blocking ET_A receptors in mammals, was unexpectedly found to be com-

pletely ineffective in preventing the effects of ET-1. This may possibly be due to the fact that the receptors of fish have a very different molecular configuration from those of mammals, although receptors in both classes of vertebrates might have a common ancestor that existed more than 400 million years ago. Incidentally, it is possible that the receptors in fish might more closely resemble the original ancestral receptors than those in mammals. In any case, a complete understanding of the interaction between ETs and their receptors in vertebrates must await future determinations of their respective molecular structures.

Once it had reached a maximum, the extent of pigment aggregation induced by ET-1 gradually decreased. The phenomenon can be ascribed to so-called "down regulation", although further studies are needed for a complete explanation of the sequence of events.

With respect to the mechanism of signal transduction as it relates to the action of ET-1 on vascular smooth muscle cells of mammalian systems, production of inositol 1,4,5-trisphosphate (IP_3) by activated phospholipase C, elevation of the intracellular concentrations of Ca^{2+} ions, activation of protein kinase C, and so on, have all been suggested as possible factors [17]. In the pigment-aggregating response to norepinephrine of the melanophores in the Nile tilapia *Orechromis niloticus*, the involvement of the PI response has recently been demonstrated [8]. Therefore, it seems quite probable that a mechanism similar to that involved in the action of ET on mammalian smooth muscles or similar to that involved in the action of norepinephrine on melanophores of fish is operative in the response of melanophores to ET-1. Studies to examine these possibilities are in progress.

ACKNOWLEDGMENTS

We heartily thank Dr. M. Yano, Tsukuba Research Institute of the Banyu Pharmaceutical Company, Tsukuba, Ibaraki, for the generous gift of BQ-123. Our thanks are also due to Drs. N. Oshima and M. Sugimoto of this laboratory for their critical comments, and to Drs. I. Takayanagi and K. Koike of the Department of Pharmacology, School of Pharmaceutical Science of this University, for invaluable advice. This work was supported by grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 Fujii R (1959) Mechanism of ionic action in the melanophore system of fish I. Melanophore-concentrating action of potassium and some other ions. Annot Zool Japon 32: 47–59

2 Fujii R (1993) Cytophysiology of fish chromatophores. Int Rev Cytol 143: 191–255

3 Fujii R, Miyashita Y (1975) Receptor mechanisms in fish chromatophores—I. Alpha nature of adrenoceptors mediating melanosome aggregation in guppy melanophores. Comp Biochem Physiol 51C: 171–178

4 Fujii R, Miyashita Y (1976) Receptor mechanisms in fish chromatophores—III. Neurally controlled melanosome aggregation in a siluroid (*Parasilurus asotus*) is strangely mediated by cholinoceptors. Comp Biochem Physiol 55C: 43–49

5 Fujii R, Novales RR (1969) The nervous mechanism controlling pigment aggregation in *Fundulus* melanophores. Comp Biochem Physiol 29: 109–124

6 Fujii R, Oshima N (1986) Control of chromatophore movements in teleost fishes. Zool Sci 3: 13–47

7 Fujii R, Miyashita Y, Fujii Y (1982) Muscarinic cholinoceptors mediate neurally evoked pigment aggregation in glass catfish melanophores. J Neural Transmission 54: 29–39

8 Fujii R, Wakatabi H, Oshima N (1991) Inositol 1,4,5-trisphosphate signals the motile response of fish chromatophores—I. Aggregation of pigment in the tilapia melanophore. J Exp Zool 259: 9–17

9 Hayashi H, Fujii R (1993) Muscarinic cholinoceptors that mediate pigment aggregation are present in the melanophores of cyprinids (*Zacco* spp.). Pigment Cell Res 6: 37–44

10 Iga T, Matsuno A (1980) Scale melanophores of *Zacco temmincki*; a preparation suitable for physiological or pharmacological studies on fish melanophores. Zool Mag (Tokyo) 89: 227–234

11 Iga T, Takabatake I (1982) Denervated melanophores of the dark chub *Zacco temmincki*: method of denervation and the evaluation of preparations for physiological experiments. Annot Zool Japon 55: 61–69

12 Ihara M, Noguchi K, Saeki T, Fukuroda T, Tsuchida S, Kimura S, Fukami T, Ishikawa K, Nishikibe M, Yano M (1991) Biological profiles of highly potent novel endothelin antagonist selective for the ET_A receptor. Life Sci 50: 247–255

13 Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T (1989) The human endothelin family: three structurally and pharmaco-

logically distinct isopeptides predicted by three separate genes. Proc Nat Acad Sci USA 86: 2863–2867

14 Kasukawa H, Fujii R (1985) Receptor mechanisms in fish chromatophores—VII. Muscarinic cholinoceptors and alpha adrenoceptors, both mediating pigment aggregation, strangely coexist in *Corydoras* melanophores. Comp Biochem Physiol 80C: 211–215

15 Kasukawa H, Oshima N, Fujii R (1986) A comparative survey on the type of sympathetic neuromelanophore transmission in catfishes. Comp Biochem Physiol 85C: 115–120

16 Kasuya Y, Kobayashi H, Uemura H (1991) Endothelin-like immunoreactivity in the nervous system of invertebrates and fish. J Cardiovascular Pharmacol 17: S463–S466

17 Little PJ, Neylon CB, Tkachuk VA, Bobik A (1992) Endothelin-1 and endothelin-3 stimulate calcium mobilization by different mechanisms in vascular smooth muscle. Biochem Biophys Res Commun 183: 694–700

18 Masaki T, Yanagisawa M, Goto K, Kimura S, Takuwa Y (1991) In "Cardiovascular Significance of Endothelium-derived Vasoactive Factors" Ed by GM Rubanyi, Futura Publishing, New York, pp 65–81

19 Miyashita Y, Fujii R (1975) Receptor mechanisms in fish chromatophores—II. Evidence for beta adrenoceptors mediating melanosome dispersion in guppy melanophores. Comp Biochem Physiol 51C: 179–187

20 Olson KR, Duff DW, Farrell AP, Keen J, Kellogg MD, Kullman D, Villa J (1991) Cardiovascular effects of endothelin in trout. Am J Physiol 260: H1214–H1223

21 Oshima N, Fujii R (1984) A precision photoelectric method for recording chromatophore responses *in vitro*. Zool Sci 1: 545–552

22 Poder TC, Silberberg SD, Rampe D (1991) Contraction of reptile, amphibian, and fish blood vessels by endothelin-1. Can J Pharmacol 69: 215–217

23 Sawamura T, Kimura S, Shinmi O, Sugita Y, Yanagisawa M, Goto K, Masaki T (1990) Purification and characterization of putative endothelin converting enzyme in bovine adrenal medulla: evidence for a cathepsin D-like enzyme. Biochem Biophys Res Commun 168: 1230–1236

24 Uemura H, Naruse M, Naruse K, Hirohama T, Demura H, Kasuya Y (1991) Immunoreactive endothelin in plasma of nonmammalian vertebrates. J Cardiovascular Pharmacol 17: S414–416

25 Yada Y, Higuchi K, Imokawa G (1991) Effects of endothelins on signal transduction and proliferation in human melanocytes. J Biol Chem 266: 18352–18357

26 Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411–415